Controlled Sulfatation of Natural Anionic Bacterial Polysaccharides Can Yield Agents with Specific Regenerating Activity in Vivo

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The regenerating activities of chemically modified anionic bacterial polysaccharides by O sulfonation were investigated using a in vivo model of rat injured muscle regeneration. Glucuronan (GA), a linear homopolysaccharide of →4)-β-d-Glc*p*A-(1→ residues partially acetylated at the C-3 and/or the C-2 position, and glucoglucuronan (GGA), a linear heteropolysaccharide of →3)-β-d-Glc*p*A-(1→4)-β-d-Glc*p*-(1→ residues were sulfated. SO_3-DMF sulfatation complex provided polysaccharides with different sulfur contents, however, a depolymerization occurred because we did not use large excess of pyridine to obtain pure modified polysaccharides. A regenerating activity on injured *extensor digitorum longus* (EDL) muscles on rats was obtained with these two sulfated anionic polymers. The position of sulfate groups on glucoglucuronan (primary or secondary alcohol) seems to have no influence on the biological activity by opposition to the degree of sulfatation both for the glucuronans and the glucoglucuronans. The yield of acetate groups in the glucuronan polymer modulated the specific activity.

Introduction

A growing body of evidence attributes to proteoglycans (PGs) and their glycosaminoglycans moieties (GAGs) a key role in the control of growth factors activities through specific interactions. Associated with the extracellular matrix and cell membranes, GAGs and moreover heparan sulfates (HS) interact with "heparin binding growth factors" (HBGFs) and serve as storage sites for these factors. When a tissue is injured, PGs are degraded by enzymes activities and HBGFs became bioavailable. These factors can then trigger cellular migration, proliferation, differentiation, and associated neoangiogenesis, all steps necessary for tissue repair. HBGFs are also submitted to proteolysis and their degradation interferes with the repair process. Compared to the main approches focusing on the delivery of growth factors (GFs) to compensate their deficiency, we opted for a radically different strategy.[1]

Several polymers mimicking HS for their ability to interact with and protect HBGFs[2] have been shown to stimulate healing of damaged tissues in various in vivo models.[3−7] These polymers designated as regenerating agents (RGTAs) may increase HBGFs bioavailability favoring tissue repair.

RGTAs included various families of water soluble polymers including carboxymethylated dextran sulfate derivatives[8,9] and a family of polyesters derived from malic acid containing sulfonates and other functional pendant groups.[10]

In the case of dextran derivatives, glucose residues are mainly etherified by one carboxymethyl group mainly on C2, although some residues may be etherified by two carboxymethyl groups on C2/C4, C2/C3, and C3/C4 positions.[8]

In previous works, we described the production by *Rhizobia* strains of a glucuronan partially acetylated composed of [→4)-β-d-Glc*p*A-(1→] [11] and of a glucoglucuronan composed of [→3)-β-d-Glc*p*A-(1→4)-β-d-Glc*p*-(1→].[12] The original structure of these anionic bacterial polysaccharides, consisting of a regular sequence of uronic acid and neutral sugar or of partially O-acetylated uronic acid, allowed us to study the influence of the position and the degree of sulfatation as well as the contribution or not of acetate groups on the capacity to stimulate tissue regeneration. Structural data of sulfated glucuronans and sulfated glucoglucuronans were obtained by spectroscopic analysis.

From a biological point of view, these various sulfated polymers were evaluated in terms of tissue regeneration in a crushed extensor digitorum longus (EDL) muscle, using a previously reported dextran based RGTA RG1503 as a positive control on the in vivo experiments.[8] Sulfatation

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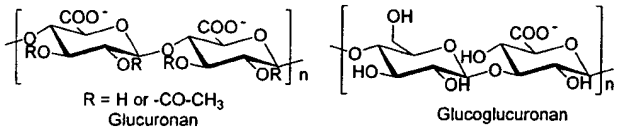
Scheme 1. Schematic Representation of Glucuronan and Glucoglucuronan



reactions were first conducted with the commonly used SO_3−pyridine complex. The resulting product tended to be colored brown[13] and exhibit a toxic effect in the in vivo assays. This toxicity seemed to be linked to the presence of a pyridinium contaminant. Thus, we report the use of the SO_3−dimethylformamide complex to obtain pure sulfated polysaccharide which stimulated tissue regeneration and were devoided of side effects.

Experimental Section

Materials and General Methods. Sulfur trioxide pyridine or dimethylformamide complexes, pivaloyl chloride and tetrabutylammonium hydroxyde, were from Sigma-Aldrich Chimie S.arl, France. DMSO, DMF were distilled before used. ^1H and ^{13}C NMR spectra were recorded with a Bruker Avance 300 MHz spectrometer from samples in D_2O at 80 °C using sodium tetramethylsilyl-propionate (TMSP) as an external standard. Absolute determination of molecular weights and size distributions were performed on polysaccharide solutions by size exclusion chromatography (SEC) eluted in 0.1 M $LiNO_3$ coupled to a multi-angle laser light-scattering photometer (MALLS; Dawn DSP-F, Wyatt Technology) connected in series to a differential refractive index detector (ERC 7515A, Erma Cr. Inc.). A set of columns SB-804-HQ and SB-806-HQ (from Shodex) were used for GA and GGA analysis, whereas a TSK Gel G3000 PWXL (Toso Haas) column was used for GA and GGA sulfated derivatives. The degree of substitution on sulfate groups (dsS), defined as the number of sulfate groups by disaccharide unit, was determined at 25 °C on protonated samples (7 mg/mL) by conductometric titration performed with a conductimeter Tacussel CD 810 by addition of NaOH 0.2 M.

Polysaccharides Production. Partially acetylated anionic bacterial glucuronan (GA) and glucoglucuronan (GGA) (Scheme 1) are excreted respectively during growth of *Sinorhizobium meliloti* M5N1 CS (NCIMB 40472)[11] and *Rhizobium sp* T1 strains.[12] The strains were cultivated 75 h in 20-liter reactors containing 15 liters of RCS medium,[14] and then bacteria were removed from the culture media by centrifugation (33 900 × g, 40 min). Purifications were performed by successive ultrafiltrations. The high molecular weight polysaccharides were concentrated from the cell-free broth by tangential ultrafiltration on a 100 000 normal-molecular-weight cutoff (NMWCO) polysulfone membrane (0.1 m^2) from Sartorius (Göttingen, Germany). After a first concentration, the 100 kDa retentate was diluted with one volume of distilled water and purified by ultrafiltration with the same membrane as previously; this step was repeated five times. The medium molecular weight polysaccharides were extracted from the 100 kDa filtrate by tangential ultrafiltration on a 50 000 NMWCO membrane. The low molecular weight polysaccharides contained in the 50 kDa filtrate were then purified by tangential ultrafiltration on a 20 000 NMWCO membrane.

The polymer fractions (20 < Mw <50 kDa, 50 < Mw < 100 kDa and 100 kDa < Mw) were dehydrated by lyophilisation.

Dextran Based Polysaccharides. Dextran based polysaccharide RG1503 used as a control for in vivo experiments was obtained from Caruelle et al (Créteil, France). The RG1503 carboxylation and O-sulfonation degrees (determined by glucosidic unit) were respectively 0,26 and 1,92.[8]

Glucuronan Partial Deacetylation. A solution of glucuronan (1 g/L) was adjusted to pH 11 by addition of 4 M NaOH and stirred 6 h at 30 °C. Then the reaction mixture was cooled at room temperature, neutralized by addition of 2 M HCl, and dialyzed against deionized water. The solution was freeze-dried to give the partially acetylated glucuronan GA0.4Ac.

Tetrabutylammonium Polysaccharide Salt Obtention. An aqueous solution of glucuronan (1 g/l) was dialyzed (cutoff membrane from 12 000 to 14 000 Da) against a 2.5 mM HCl solution. The glucuronan was then broated to pH 7.3 by tetrabutylammonium hydroxyde ($tBuN^+$ OH^-, from Sigma). The tetrabutylammonium glucuronate salt (GAtBuN) was dried by lyophilisation.

An aqueous solution of glucoglucuronan (10 g/L) was reacted with a acidified sulfonic resin, then neutralized by tetrabutylammonium hydroxyde, and dried as previously to give the GGAtBuN.

Polysaccharides O Sulfonation. The GAtBuN (1.5 g) was dissolved in 30 mL of DMSO and stirred at 40 °C overnight. A SO_3−pyridine complex (1.5 g) was added, and the reaction mixture was stirred at 40 °C for 60 min. The sulfated polysaccharide was precipitated with ethanol (10 volumes). The precipitate was then dissolved in 20 mL of 1 M NaCl, and the pH was adjusted to 7 with 2 M NaOH. The sulfated polysaccharide was collected by precipitation with ethanol as previously and was purified by dialysis first against 0.1 M NaCl, to ensure a complete exchange of the residual $tBuN^+$ by Na^+ ions, then against water. The final product was dried as previously.

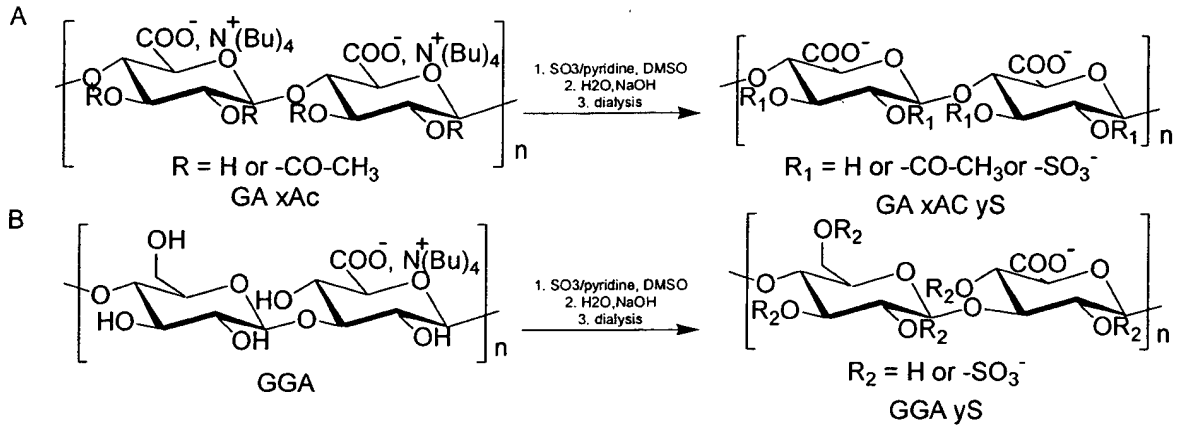
The same procedure was performed on the partially deacetylated glucuronan and on the glucoglucuronan.

Selective Sulfatation of Glucoglucuronan on Secondary Alcohol Functions. The GGAtBuN obtained as aboved (1.5 g) was dissolved in anhydrous DMF (20 mL), pivaloyle chloride (1.3 mL) was added, and the mixture was maintained 1 h at 40 °C. The polymer was collected by precipitation with ethanol and dried under vacuum. ^{13}C NMR spectroscopy was used to control the substitution by the pivaloyl group of the 6-hydroxyl group in glucose residues.

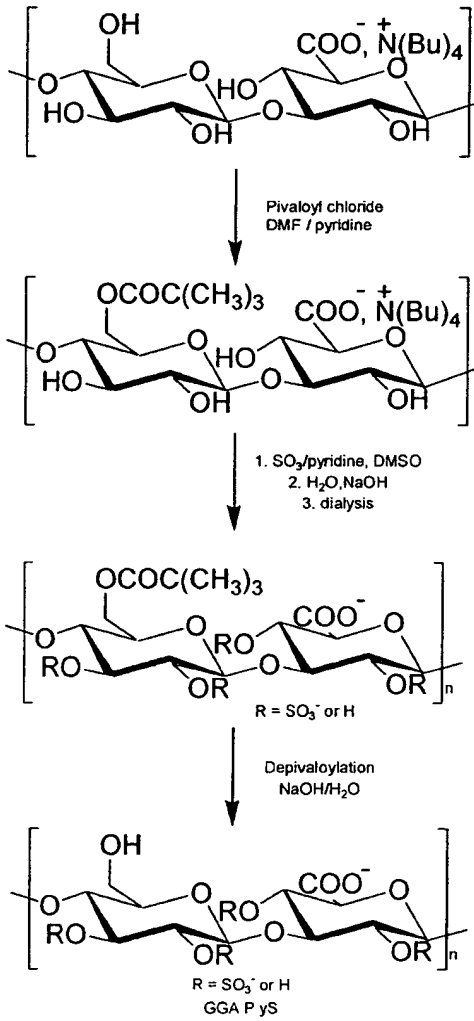
The sulfatation procedure described above was applied on the 6-piva-glucoglucuronan tetrabutylammonium salt. Then, the polymer depivaloylation was performed at pH 12 with 2 M NaOH at room temperature during 4 h. The sulfated polymer noted GGA P xS was collected and dried as previously.

Muscle Regeneration Test. Muscle crushing was performed as previously described.[6] Briefly, two months old

Scheme 2. Synthetic Route of Glucuronan (A) and Glucoglucuronan (B) Sulfatation by SO_3–Pyridine Complex



Scheme 3. Synthetic Route of Glucoglucuronan Sulfatation on Secondary Alcohol Groups



male rats Wistar (300 g) were anaesthetized with ether. The EDL muscles of the legs back were mechanically injured with a Pean grip maintained during 15 s. 100 μL of polymers dissolved in phosphate buffer saline (PBS, 200 μg/mL) were injected into the injured muscle. The control consisted of animals treated by injection of PBS (100 μL). Two animals were tested for each polymer. After a 8 days period, the muscles were taken and immediately frozen in isopentane cooled by liquid nitrogen. Samples sections of 10 μm thick were extemporaneously stained with Gomori's trichrome and examined with light microscopy.

Table 1. Sulfatation Conditions of Glucuronans 0.4 Ac and 1.4 Ac, Glucoglucuronans with or without Protection by Pivaloyl Groups and Analysis of the Sulfated Products

sample	SO_3–pyr[a] (equiv)	dsS [b]	$\overline{Mn}$[c] (Da)	$\overline{Mw}$[c]
GA1.4Ac			193 000	215 000
GA1.4Ac1.3S	4	1.3	9200	12 500
GA1.4Ac1.2S	8	1.2	7900	10 600
GA1.4Ac1.4S	12	1.4	5200	14 400
GA0.4Ac			158 000	189 000
GA0.4Ac1.6S	12	1.6	6600	15 800
GGA			90 000	550 000
GGA0.2S	1	0.2	41 000	136 000
GGA1S	2	1.0	39 000	112 000
GGA1.7S	4	1.7	6400	8,300
GGA1.9S	6	1.9	6200	8300
GGAP1.2S	6	1.2	14 800	28 000

[a] The SO_3–pyridine complex was equivalent to a dissaccharide unit. [b] Calculated by conductometric analysis. [c] Determined by SEC/MALLS.

Results

Determination of Glucuronans Acetylation Degrees. GA degree of substitution by acetyl groups on a disaccharide unit determined by ^{1}H NMR spectroscopy as previously described[15] was 1.4 and 0.4 for respectively the native glucuronan noted GA1.4Ac and the partially deacetylated glucuronan noted GA0.4Ac.

Synthesis and Structure Analysis of Polymers Sulfated with the SO_3–Pyridine Complex. The chemical sulfation procedure using the SO_3–pyridine complex was applied on GA1.4Ac and GA0.4Ac and GGA tetrabutylammonium polymer salts according to the synthetic route represented in Scheme 2, parts A and B. The same sulfation procedure was applied on the 6-piva-glucoglucuronan tetrabutylammonium salt (Scheme 3).

Different SO_3–pyridine complex concentrations were used in order to obtain various GA and GGA sulfated polymers. The results of the sulfatations are summarized in Table 1. The degree of sulfatation (dsS) estimated by conductometric analysis was in the range 0.2–1.9. The highest dsS obtained

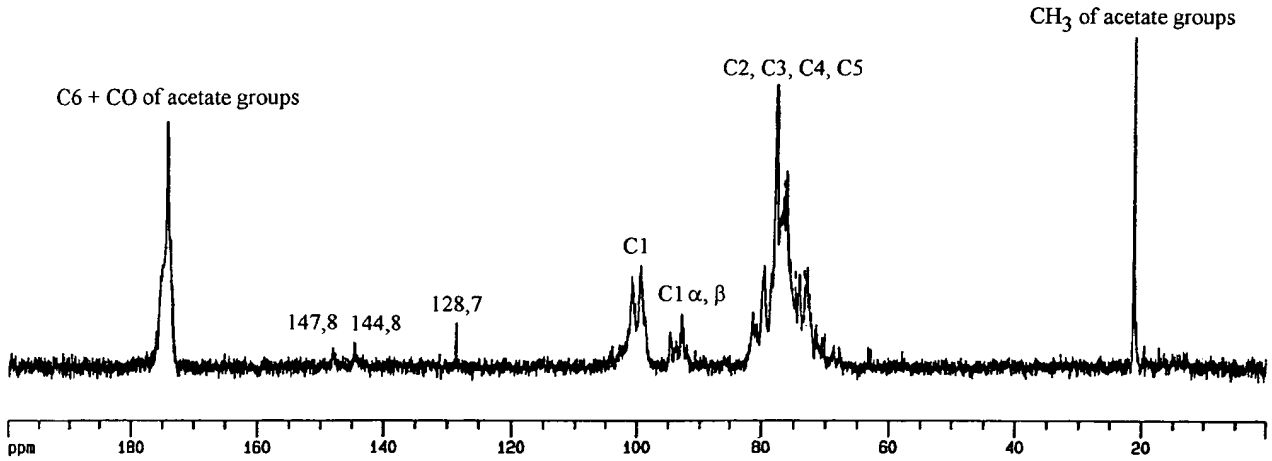


Figure 1. Typical ^{13}C NMR spectra of sulfated glucuronan by the SO_3−pyridine complex.

for the GA1.4Ac derivatives was 1.4 despite the excess of sulfating reagent used (equal to 12 SO_3-pyridine equivalent by disaccharide unit). However a dsS of 1.6 was obtained on the less acetylated glucuronan GA0.4Ac.

We noted that for a similar sulfating reagent concentration applied (4 eq) the sulfatation of GGA samples was higher those obtained for the GA samples and that the pivaloylation of primary alcool groups in GGA reduced the polymer sulfatation.

In all cases, GA and GGA backbones were partially degrade and the products showed number average molecular weights ($\overline{Mn}$) lower than the precursors. Increasing the amounts of SO_3−pyridine complex gave polysaccharides with relatively high contents of sulfate groups, but it caused a decrease in the molecular weight.

C^{13} NMR spectra analyses performed on the sulfated GA polymers revealed the presence of signals between 90 and 95 ppm characteristics of C1-β and C1-α (Figure 1). The detection of signals from anomeric carbons on the sulfated polymers was significant for polymer degradation occurring during the sulfatation procedure. This result was in agrement with those obtained by SEC analysis (Table 1).

A yellow coloration observed on the sulfated polysaccharides lead us to suspect the presence of pyridine in the SO_3−pyridine complex treated polymers. ^{13}C NMR spectra of related GA and GGA polymers revealed the presence of small signals at 147.8, 144.8, and 128.7 ppm characteristic of pyridinium (Figure 1). These values are relatively close to those reported on the literature for the pyridinium salt (148.4, 142.5, and 129 ppm). Pyridinium content was determined by UV spectroscopy (210−350 nm) by using pyridinium solutions as reference. UV spectra of the glucuronan and glucoglucuronan sulfates revealed 3 peaks in the region 250−265 nm. Higher contamination was observed in polymers with higher sulfate contents (data not shown). Different purification procedures, as dialysis under basic conditions or anion exchange chromatography, were unsuccessful to eliminate pyridinium residues. Such results can be compared to those described by De Belder[16] who reported that pyridinium residues are readily introduced at the reducing end-groups of the polysaccharide chains when a polysaccharide in solution is treated with a sulfonic acid reagent or with SO_3 using pyridine as a solvent.

According to previous data,[17] the major signals observed on the GGA0.2S ^{13}C NMR spectrum (Figure 2b) corresponded to carbons from glucose and glucuronic acid residues. However, a small signal at 67 ppm was detected on the GGA0.2S spectrum. This signal was higher on the GGA1S ^{13}C NMR spectrum (Figure 2a). Concomitantly with the increase of the signal at 67 ppm, we noted a significant reduction of the C6 glucose signal. This result lead us to assign the signal at 67 ppm to C6 in glucose residues sulfated at C6. As the major signals on GGA1S spectrum correspond to those assigned to C2, C3, C4, and C5 of native GGA, we concluded that the GGA 1S polymer was mainly sulfated on the primary alcohol on the glucose residue, and this result agrees with those described by Gao et al.[18]

These results indicated that the sulfatation procedure using the SO_3−pyridine complex was convenient to obtain glucuronans and glucoglucuronans with various dsS. However, significant polymer degradation was detected related to the sulfatation conditions, and more so the presence of pyridinium substituents was considered as a major drawback for biological tests. So further experiments were performed in order to obtain pure sulfated polysaccharides with higher molecular weights.

Synthesis and Structure Analysis Polymers Sulfated with the SO_3−DMF Complex. Two glucuronan samples, GA 1.4Ac and the GA 0.4Ac, and two glucoglucuronan samples, the native GGA and the 6-piva-glucoglucuronane, were sulfated with the SO_3−DMF complex instead of the SO_3−pyridine complex. The synthetic route was similar those describe Schemes 2 and 3.

Polysaccharide sulfation with the SO_3−DMF complex, known to be more reactive than the SO_3−pyridine one, lead to dsS higher than those obtained with the pyridine complex for the same number of equivalent added (Table 2). We noted the sulfatation procedure using the SO_3−DMF complex was less degrading than those using the SO_3−pyridine complex, where the polysaccharides $\overline{Mn}$ were between 6- and 12-fold higher.

^1H NMR spectra obtained from sulfated glucuronan polymers (data not shown) revealed that the degree of substitution by acetyl residues remained constant whatever the sulfatation conditions applied.

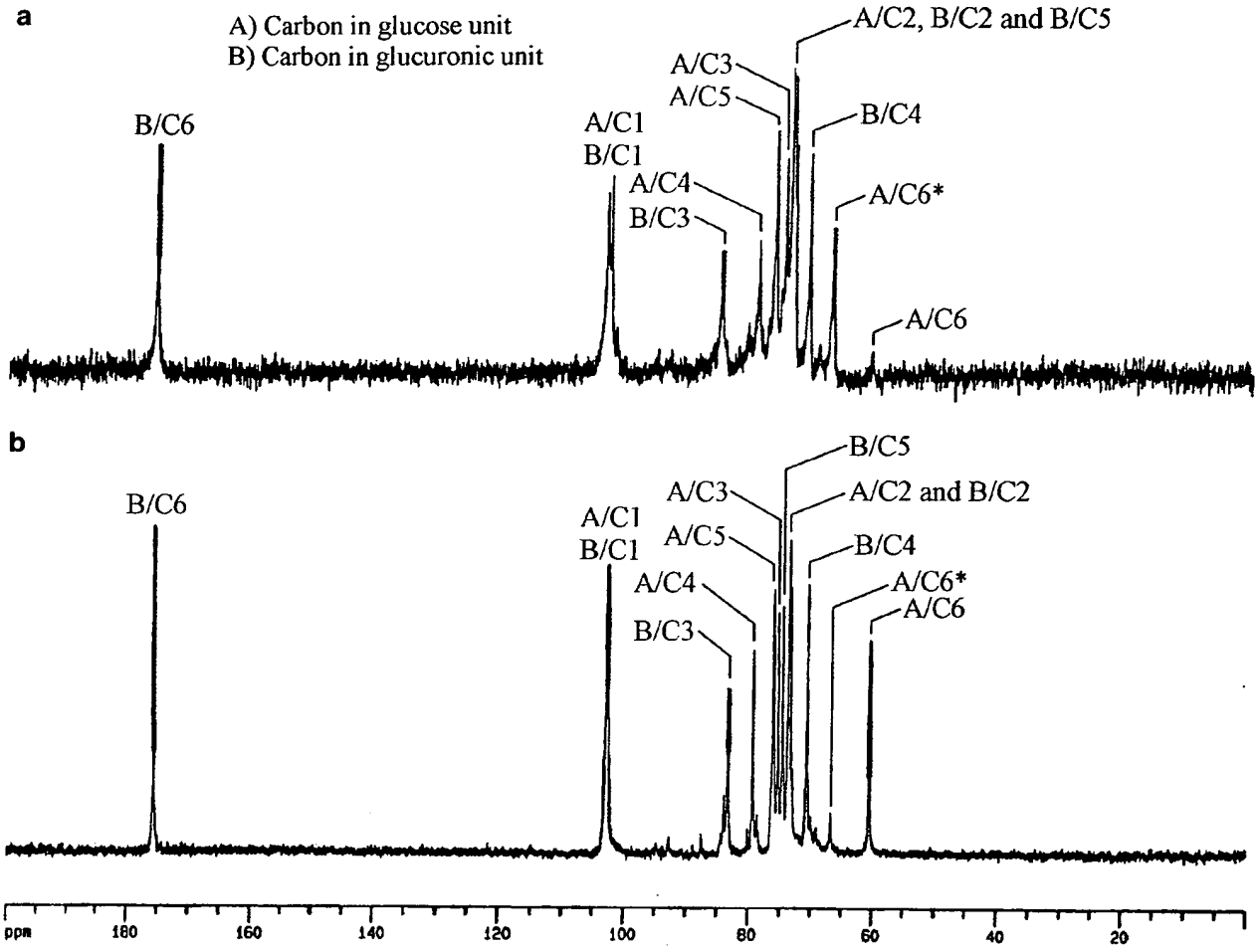


Figure 2. ^{13}C NMR spectra of sulfated glucoglucuronans by the SO_3–pyridine complex (a) GGA 1S; (b) GGA 0.2S. A/C1: C1 of β-d-Glc*p* residues; B/C1: C1 of β-d-Glc*p*A residues; A/C2: C2 of β-d-Glc*p* residues; B/C2: C2 of β-d-Glc*p*A residues; A/C3: C3 of β-d-Glc*p* residues; B/C3: C3 of β-d-Glc*p*A residues; A/C4: C4 of β-d-Glc*p* residues; B/C4: C4 of β-d-Glc*p*A residues; A/C5: C5 of β-d-Glc*p* residues; B/C5: C5 of β-d-Glc*p*A residues; A/C6: C6 of β-d-Glc*p* residues; A/C6*: C6 of the 6-*O*-sulfo-β-d-Glc*p* residues; B/C6: C6 of β-d-Glc*p*A residues.

Table 2. Sulfatation Conditions (by the SO_3–DMF Complex) of Glucuronans 0.4 Ac and 1.4 Ac, Glucoglucuronans with or without Protection by Pivaloyl Groups, and Analysis of the Sulfated Products

sample	SO_3–DMF[a] (equiv)	dsS [b]	$\overline{Mn}$[c] (Da)	$\overline{Mw}$[c] (Da)
GA1.4Ac1.7S	6	1.7	54 500	92 000
GA0.4Ac2.6S	6	2.6	59 000	92 000
GGA1.8S	4	1.8	56 000	270 000
GGA3.2S	6	3.2	79 500	285 000
GGAP2S	5	2.0	66 200	331 000

[a] The SO_3-DMF complex was equivalent to a dissaccharide unit. [b] Calculated by conductometric analysis. [c] Determined by SEC/MALLS.

Comparison between ^{13}C NMR spectra from GA1.4Ac, GA1.4Ac1.7S, and GA0.4Ac2.6S, sulfated with the SO_3–DMF complex (Figure 3) revealed modifications in the region 100–105 and 65–85 ppm.

The glucuronan contains six residues: 2-*O*-Ac-β-d-Glc*p*A (residue A), 3-*O*-Ac-β-d-Glc*p*A (residue B), 2,3-*O*-Ac-β-d-Glc*p*A(residue C) and three unacetylated residues, D, following 2-*O*-Ac-β-d-Glc*p*A residues, E, preceding 3-*O*-Ac-β-d-Glc*p*A residues, and F, a set of unacetylated residues. Peak assignments (Figure 3c) were made from previous two dimensionnal NMR measurement.[19]

The signals relative to C-1 from unacetylated residues D, E, and F were shifted to 100.80 and 100.02 ppm. The difference of intensity between GA1.4Ac1.7S and GA0.4Ac2.6S might be mainly attributable to a different degree of substitutions of sulfate groups in the polymer backbone. The signals corresponding to C-3 (75.54 ppm), C-4 (82.05 ppm), and C-2 (73.98 ppm) from nonacetylated residues show variation of intensity or little variation of chemical shifts, which indicate that a substitution occurs on non acetylated residues.The signal at 76.41 corresponding to C-5 from glucuronic residues was still detected with a little variation of chemical shifts. New major signal appears in the region 77–81 ppm. The signals at 80.60 ppm, increasing in the highest sulfated polymers GA0.4Ac2.6S, lead us to propose that this chemical shift corresponds to the C-4 from sulfated residues in positions 2 or 3. The attribution of signals at 77.79, 78.32, and 79.33 ppm were not clear and might correspond to C-4 from acetylated residues, C-3 from 3-*O*-Ac-β-d-Glc*p*A, C-3 from unacetylted residues sulfated, or not.

These results for glucuronans sulfate indicate that the unacetylated residues were the most preferential position for sulfatation, followed by the acetylated residues.

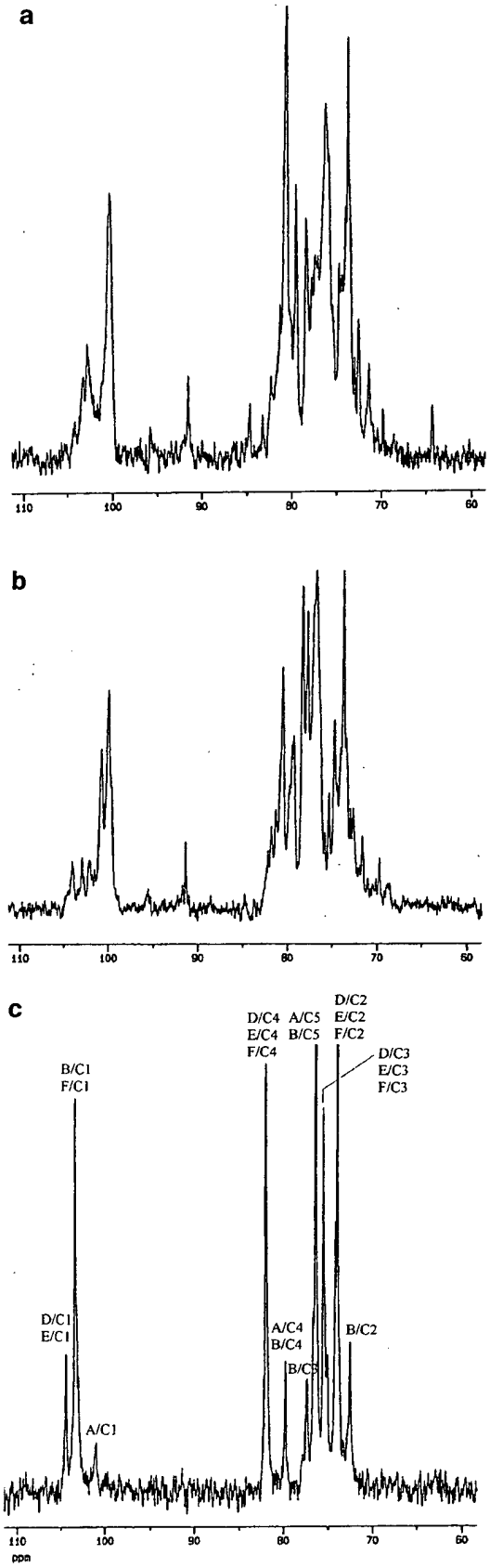


Figure 3. ^{13}C NMR spectra of the sulfated glucuronan (a) GA0.4Ac2.6S, (b) GA1.4Ac1.7S, and the precursor (c) GA1.4Ac. Residue A: 2-*O*-Ac-β-d-Glc*p*A; residue B: 3-*O*-Ac-β-d-Glc*p*A; and three unacetylated residues: D, following 2-*O*-Ac-β-d-Glc*p*A residues, E, preceding 3-*O*-Ac-β-d-Glc*p*A residues, and F, a set of unacetylated residues.

The ^{13}C NMR spectrum from GGA1.8S revealed important modifications in the region 65−85 ppm, although new and expended signals were observed indicating that sulfatation of secondary alcohol functions on glucose and glucuronic residues takes place without specificity of position (Figure 4).

^{13}C NMR spectra from the sulfated glucoglucuronan GGA P 2S (Figure 5) revealed a signal at 67 ppm observed previously with GGA not protected on C6. This signal first attributed to C6 sulfated glucose residues indicates a non complete pivaloylation. The two signals at about 97 and 105 ppm in the anomeric region of C1 were not detected on the ^{13}C NMR spectrum from GGA1.8S. Sulfatation of the secondary alcohol was lower on GGA1.8S than on GGA P2S. The two signals detected at 97 and at 105 ppm indicate the presence of two different sulfated residues; however, at this level of experimentation, it was not possible to determine if sulfatation was mainly on C2 or C3 in glucose or on C2 or C4 in glucuronic residues. The non visualization of the specific signals on the GGA1.8S ^{13}C NMR spectrum was probably due to a low proportion of the different sulfated residues in the conditions applied.

Effect of Sulfation on Regenerating Activities. Sulfated and nonsuflated polysaccharides were tested in the muscular model of regeneration corresponding to EDL muscle on rats.[6] The EDL muscles were examined 8 days after injection.

Macroscopic and histological analysis of injured muscles treated by the sulfated polysaccharides contaminated by pyridinium residues not only showed poorer regeneration than PBS treated reference, but also those muscles showed to be invaded by mononucleated cells comparable to inflammatory cells. A large area of necrosis represented by pseudo myelinic rolling up of cells on themselves indicated global muscle degeneration.

Histological sections of native glucuronan and glucoglucuronan injected muscles compared to controls consisting of RGTA RG1503 (200 μg/mL) or PBS (Figure 6) revealed no difference between the PBS and the GGA treated muscles demonstrating no intrinsic toxicity. However, a weak regenerating activity was observed on GA native treated muscles (data not shown). To test the influence of the polysaccharide molecular weight on its regenerating activity, two native GA polymer fractions ranging between 20 < Mw <50 kDa and 50 < Mw < 100 kDa were applied on wounded EDL muscle as above. Eight days after injection, no differences were observed with the two tested native GA polymers fractions.

So the inflammatory cells observed on EDL muscles treated with the sulfated polysaccharides contaminated by pyridinium was not due to the polysaccharides backbone but to the presence of pyridinium grafted to the polymers. To determine the influence of sulfate groups substituting the anionic polymers GA and GGA, pure sulfated polymers obtained by using the SO_3−DMF complex were applied on injured muscles on rats.

Figure 7 shows histological sections of muscles treated with sulfated glucuronans GA1.4Ac1.7S. Good regenerating activity was observed and a few myotubes among proliferating mononucleated cells were detected. These results were similar as those observed with RGTA RG1503, a carboxym-

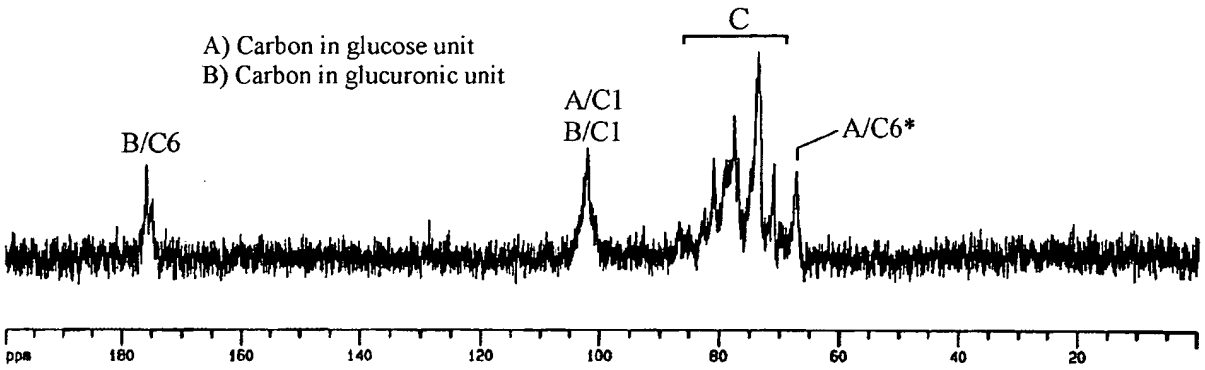


Figure 4. ^{13}C NMR spectra of sulfated glucoglucuronans GGA1.8S. A/C1: C1 of β-d-Glc*p* residues; B/C1: C1 of β-d-Glc*p*A residues; A/C6*: C6 of the 6-*O*-sulfo-β-d-Glc*p* residues; B/C6: C6 of β-d-Glc*p*A residues; C: C2, C3, C4 and C5 of β-d-Glc*p* and β-d-Glc*p* residues.

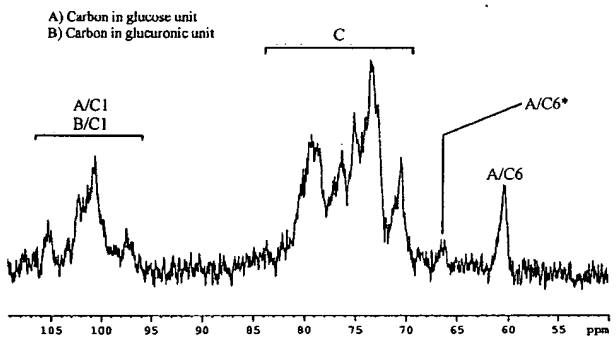


Figure 5. ^{13}C NMR spectra (region 50−110 ppm) of the sulfated glucoglucuronan GGA P 2S. A/C1: C1 of β-d-Glc*p* residues; B/C1: C1 of β-d-Glc*p*A residues; A/C6: C6 of β-d-Glc*p* residues; A/C6*: C6 of the 6-*O*-sulfo-β-d-Glc*p* residues; C: C2, C3, C4 and C5 of β-d-Glc*p* and β-d-Glc*p* residues.

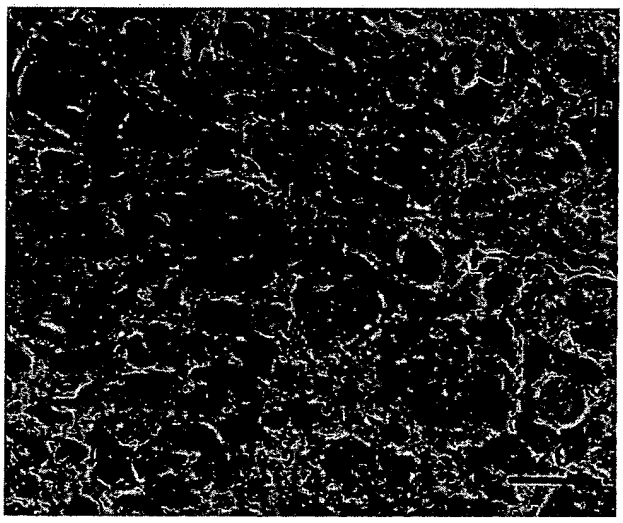


Figure 6. Large magnification (× 600) of a Gomori-stained transverse section of the mid portion of nontreated EDL. After 7 days, only few and small regenerated myotubes are surrounded by many mononucleated cells. The architecture is not yet organized.

ethylated and sulfated dextran.[8] However, the regenerating activity was lower than the most efficient dextran derivatives containing either carboxymethyl, benzylamide, or sulfate groups. In the conditions tested, GA1.4Ac1.7S enhanced the regenerating activity, expressed by an enhanced myotubes formation. Experiments performed with the sulfated and partially deacetylated glucuronan GA0.4Ac2.6S revealed a weaker regenerating activity than GA1.4Ac1.7S: very few regenerated myotubes were observed to be sparse among

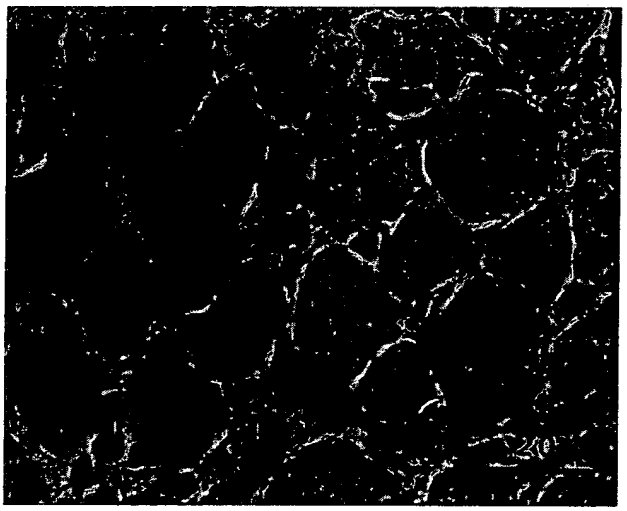


Figure 7. Large magnification (× 600) of Gomori-stained transverse section of the mid portion of GA1.4Ac1.7S-treated EDL. At the same time, a single GA1.4Ac1.7S-treatment stimulated regeneration and architecture myofibers, respectively.

numerous mononucleated cells. From these results, we propose that the GA regeneration activity is induced not only by the presence of sulfate groups, allowing specific interaction with the matrix cells components, but also by hydrophobic groups like acetyl groups. This result can be compared to those obtained with dextran derivatives containing a benzylamide group.

As for the sulfated GA polymers, the dsS of GGA influenced EDL muscle regeneration. When regioselective sulfatation was performed on the GGA, the GGA P2S product exhibited the same biological activity as the GGA1.8S product sulfated basically on primary alcohol functions. The best regenerating activities were obtained with higly sulfated GGA3.2S. The positions of sulfate groups did not show an influence on the polysaccharide regenerating activity on EDL muscle.

In conclusion, sulfation of GGA and GA afford the first family of microbial polysaccharides including GA1.4Ac1.7S and GGA3.2S exhibiting specific biological activities on EDL muscle, similar to those obtained with RGTA RG1503. The position of sulfate groups on GGA derived polymers seems to have no influence on the regenerating activity by opposition to the degree of sulfatation. Further experiments will determine the influence of hydrophobic groups grafted on sulfated GGA polymers. Another point consists of

controlling the degradation of the molecular backbone, but without using molecules such as pyridine that contaminate the resulting products.

These studies correspond to a new approach for studying the relation between the sequence of anionic polysaccharide GA or GGA, the rate of sulfate and hydrophobic grafted groups, and the regenerating activity by comparison with heparan sulfates. The biological effect of these modified bacterial polysaccharides could be to some extent explained by their partial resistance against natural enzymes which degrade mammalian heparan sulfates. Preliminary results obtained with RGTA RG1503 might approved this hypothesis.

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